D3

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00 — A1

(11) International Publication Number: WO 97/47325

(43) International Publication Date: 18 December 1997 (18.12.97)

(21) International Application Number: PCT/SE97/01019

(22) International Filing Date: 11 June 1997 (11.06.97)

(30) Priority Data:
9602300-7 11 June 1996 (11.06.96) SE

(71) Applicant *(for all designated States except US)*: KAROLINSKA INNOVATIONS AB [SE/SE]; Doktorsslingan 23, S-171 77 Stockholm (SE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: PETTERSSON, Sven [SE/SE]; Centrum för Bioteknik, Novum, Karolinska Institutet, S-141 57 Huddinge (SE). NEURATH, Markus, F. [DE/DE]; Lab. of Immunology, University of Mainz, Langenbeckstrasse 1, D-55101 Mainz (DE). MEYER ZUM BÜSCHENFELDE, K-H [DE/DE]; Lab. of Immunology, University of Mainz, Langenbeckstrasse 1, D-5501 Mainz (DE). STROBER, Warren [US/US]; National Institute of Health, 10, Center Drive, MSC 1890, Building 10, Room 11N, 238, Bethesda MD 20892 (US).

(74) Agents: ONN, Thorsten et al.; AB Stockholms Patentbyrå, Zacco & Bruhn (publ), P.O. Box 23101, S-104 35 Stockholm (SE).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NEW COMPOSITION AND METHODS

(57) Abstract

Local administration of p65 antisense oligonucleotides is shown to be an effective way to downregulate the expression of cytokines in inflammatory conditions, e.g. in Crohn's disease or rheumatoid arthritis. A single local administration of p65 antisense oligonucleotides is more effective than single or daily administration of glucocorticoids. Additionally local administration of p65 antisense oligonucleotides is devoid of the adverse effects associated with the systemic administration of p65 antisense oligonucleotides. Further the expression of the p65 subunit of the transcription factor NF-κB in macrophages is shown to be a useful measure in the diagnosis of inflammatory diseases, e.g. Crohn's disease and rheumatoid arthritis.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

New composition and methods

Field of the invention

The present invention relates to a new method for local treatment of inflammatory diseases, free from the drawbacks of hitherto known methods and a pharmaceutical composition for said use. The invention further relates to methods for diagnosis and treatment of inflammatory diseases and reagents, kits and compositions for such use.

Background of the invention

Inflammatory diseases, such as inflammatory diseases of the gastrointestinal tract, e.g. gastritis, colitis, Crohn´s disease, proctitis and inflammatory diseases of the skin, respiratory system and the musculosceletal system are often severely incapacitating and cause the patient great inconvenience and makes the patient susceptible to other complications. The exact mechanism behind the inflammatory response, characteristic for said group of diseases is not fully elucidated. The expression of known proinflammatory cytokines, such as IL-1, IL-6 and TNF-α is believed to play a central role in the course of development and exacerbation of said diseases.

The exact mechanism behind inflammatory reactions is yet not fully elucidated but the central role of cytokine expression is nevertheless confirmed. Present explanatory models also include an interplay between cytokines and adhesion molecules. The former can trigger the expression of the latter and vice versa. The importance of cytokines is underlined by the observation that while adhesion molecules require physical contact to exercise their influence, cytokines are present in the local fluids and have thus a broader sphere of action.

Systemic administration of p65 antisense oligonucleotides is known as a possible therapy to prevent cell adhesion. Cell adhesion is believed to be governed at least partially by the expression of the NF-κB transcription factor. Further, cell adhesion is widely recognised as one important factor in conditions such as inflammation, wound healing and tumour development. Oligomers that hybridize to a portion or part of the genes encoding the NF-κB transcription factor could thus prove useful to prevent cell adhesion. Narayanan (EP 0 589 330 A2) claims an oligomer containing or having the sequence GGG GAA CAG TTC GTC CAT GGC as useful for binding to a portion of the gene encoding the human p65 subunit.

Malthese et al. (NAR 23(1995)1143) have reported strong inhibition of cell adhesion using a p65 antisense oligonucleotide containing a G-quartet, like in the sequence claimed by Narayanan (supra). In addition, they have shown a position-independent effect of a G-quartet in their p65 antisense oligonucleotide although these mutations caused various mismatches of

the antisense oligonucleotide and the p65 translation start site. They also demonstrated by mutation analysis that the G-quartet of p65 antisense oligonucleotides is essential for activity. However, with regard to specificity of effects observed with G-quartet-containing antisense oligonucleotides, Yaswen et al. (Antisense Res. Dev. 3(1993)67) showed that such oligonucleotides cause non-specific effects on cell morphoplogy. Furthermore, it was recently shown (Burgess at al., Proc. Natl. Acad. Sci. USA, 95(1995)4051-4055) that such oligonucleotides unspecifically influence and downregulate cell adhesion. These data demonstrate that the observed effects of Malthese et al. are due to the presence of said G-quartet and not to sequence specific downregulation of p65. Furthermore, specific downregulation of p65 was also observed using G-quartet-less p65 antisense oligonucleotides (Mizoguchi et al., 258(1992)1795).

Consequently the problem of how to specifically downregulate cytokine expression in inflammatory diseases, and thus efficiently treat such diseases, remains unsolved. The hitherto used regimens; systemic administration of antisense or glucocorticoid compositions, the latter being the more common therapy, has many adverse effects. The systemic administration of antisense polynucleotides is known to cause adverse effects, in particular when the oligonucleotides penetrate the brain-blood-barrier.

The objective of the invention

The objective of the present invention is to provide a novel oligonucleotide and a pharmaceutical compound containing said nucleotide which specifically downregulates cytokine development and is suitable for the treatment and diagnosis of inflammatory diseases.

Summary of the invention

The objective of the invention is fulfilled by the specific oligonucleotide, the composition comprising said oligonucleotide and methods specified in the accompaning claims. The present inventors have surprisingly shown, that a novel pharmaceutical composition according to the appendend claims is efficient in specifically downregulating the expression of cytokines in inflammatory diseases. The novel composition is also suitable for the treatment and diagnosis of inflammatory diseases, in particular for the treatment of inflammatory diseases in the gastrointestinal tract, the skin and the nervous system. The novel oligonucleotide and composition comprising the same is particularly useful for local treatment of said diseases.

Description of the figures

Fig. 1 - Increased p65 expression in TNBS-induced colitis

a) Northern analysis for IL-1, IL-6, TNF-a and β-actin transcripts of RNA from macrophage--enriched lamina propria mononuclear cells isolated from the colon of normal SJL/J mice, ethanol-treated mice and TNBS-treated mice at day 21.

b) Gel retardation analysis with nuclear extracts from macrophage-enriched lamina propria mononuclear cells isolated from normal or TNBS-treated mice at day 21 and a NF-kB binding site. There was a striking increase in NF-kB binding capacity in TNBS-induced colitis. A super-shift after addition of 0.2 mg anti-p65 antibody (Santa Cruz, CA) was observed suggesting the presence of the p65 subunit in the NF-kB complex.

c) Western and shift-Western blotting studies for p65 expression using nuclear proteins from lamina propria macrophages isolated from normal and TNBS-treated mice at day 21. There was a striking increase of p65 levels in TNBS-induced colitis.

Fig. 2 - Specific downregulation of p65 by an antisense oligonucleotide Western blot for p50 and p65 expression by macrophage-enriched lamina propria mononuclear cells after treatment with the p65 antisense oligonucleotide and control oligonucleotides. Macrophage-enriched cells were isolated from SJL/J mice with TNBS-induced colitis at day 21 and co-incubated with 8 mM phosphorothioate oligonucleotides. A reduction of p65 expression was found in antisense-treated cells. Cell viability upon antisense-treatment was assessed by trypan blue exclusion and was always higher than 90%. p65 mRNA levels were reduced more than 90% after antisense treatment, as assessed by semiquantitative PCR (not shown).

Fig. 3 - Effects of p65 antisense treatment on TNBS-induced colitis

a) Weight changes in SJL/J mice with TNBS-induced colitis after administration of phosphorothioate oligonucleotides. Mice with TNBS-induced colitis received intravenous injection of 900 mg or local administration of 150 mg phosphorothiate oligonucleotides at day 14. Each point represents weight data from 3 mice. The standard errors are indicated. After initial reduction of the body weight in all TNBS-treated groups, the mice treated with p65 antisense oligonucleotides showed an increase in their average body weight. In contrast, no increase was observed in mice given control oligonucleotides.

b) Northern blot analysis for IL-1, IL-6, TNF-a and β-actin transcripts of RNA from macrophages isolated from the colon of mice with TNBS-induced colitis 7 days after treatment with phosphorothioate oligonucleotides. Mice with established TNBS-induced colitis

were treated locally with phosphorothioate oligonucleotides at day 14. RNA from macrophages was isolated at day 21, blotted to a nitrocellulose membrane and hybridized with specific probes. There was a striking reduction in mRNA expression of proinflammatory cytokines by macrophages in p65 antisense-treated mice.

Fig. 4 - Effect of p65 antisense treatment and glucocorticoids on TNBS-induced colitis

a) Weight changes in SJL/J mice with established TNBS-induced colitis after local administration of p65 antisense oligonucleotides or glucocorticoids. Mice with established TNBS-induced colitis were treated at day 19 after administration of TNBS when they showed about 20% weight loss. Mice were either treated by a single local administration of 150 mg p65 antisense oligonucleotides or by a single or daily local administration of 0.2 mg prednisolone. Each point represents weight data from 3 mice. The standard errors are indicated. Mice treated with p65 antisense oligonucleotides showed an increase in their average body weight that was more pronounced than the one observed in glucocorticoid--treated mice.

b) Weight changes in SJL/J mice with established TNBS-induced colitis after systemic administration of p65 antisense oligonucleotides or glucocorticoids. Mice with established TNBS-induced colitis were treated at day 19 after administration of TNBS when they showed about 20% weight loss. Mice were either treated by a single systemic administration of 900 mg p65 antisense oligonucleotides or by a single or daily systemic injection of 0.2 mg prednisolone. Each point represents weight data from 3 mice. The standard errors are indicated. Mice treated with p65 antisense oligonucleotides showed an increase in their average body weight that was more pronounced than the one observed in glucocorticoid--treated mice.

Fig. 5 - Upregulation of p65 expression in IL-10 -/- mice Western and shift-Western blotting studies for p65 expression using nuclear proteins from lamina propria macrophages isolated from IL-10 -/- mice and wild type control mice. There was a striking increase in p65 levels in IL-10 -/- mice.

Fig. 6 - Predominant role of NF-kB in chronic intestinal inflammation

The predominant role of NF-kB and p65 in TNBS-induced colitis and the colitis found in IL10 -/- mice appears to be due to its ability to regulate transcriptional activity of the promoters of proinflammatory cytokines, such as IL-1, IL-6 and TNF-a, in macrophages. While corticosteroids block NF-kB function by inducing IkBa mRNA production[42-43], p65

antisense oligonucleotides downregulate p65 mRNA levels and consecutively expression of NF-kB.

Description of the invention

The present inventors have defined a predominant role for the p65 subunit of the transcription factor NF-kB in two murine models of chronic intestinal inflammation. Furthermore, the inventors present a novel way of treating chronic colitis by local administration of p65 antisense oligonucleotides. Such treatment of chronic colitis is shown to be surprisingly more effective compared to treatment with glucocorticoids, as assessed by weight data and histologic analysis. Taken together with the finding that upregulation of p65 expression by lamina propria macrophages in patients with Crohn´s disease is associated with high production of proinflammatory cytokines, the data confirms the utility of local treatment with p65 antisense oligonucleotides in patients with this disease and similar inflammatory diseases of the gastrointestinal tract as well as other diseases associated with high production of proinflammatory cytokines.

Additionally the present inventors have shown that local NF-kB expresson correlates with rheumatoid artritis and preliminary tests in humans show, that local administration of p65 antisense oligonucleotide is a potent therapy.

The murine TNBS-model of chronic intestinal inflammation used contains several features that are consistent with those observed in Crohn´s disease in humans[1]: (i) a chronic colitis is induced that is characterized by a severe, transmural and granulomatous inflammation associated with diarrhea, rectal prolapse and weight loss; (ii) there are similarities at the T cell cytokine level since lamina propria CD4+ T lymphocytes in both diseases secrete high amounts of Th1-type cytokines, such as IFN-g, but low levels of Th2-type cytokines, such as IL-4; (iii) the present inventors have shown here that lamina propria macrophages in TNBS-induced colitis produce high levels of the proinflammatory cytokines IL-1, IL-6 and TNF-a reminiscent of the cytokine profiles previously reported in patients with Crohn´s disease[11, 12]. The elevated levels of IL-1, IL-6 and TNF-a in TNBS-induced colitis prompted us to focus in further molecular studies on the expression of NF-kB, a key transcription factor previously implicated in the transcriptional control of the promoter activity of these genes in macrophages[6, 22-23]. These studies revealed a striking overexpression of NF-kB by macrophages in TNBS-induced colitis and demonstrated that p65 is a major component of the NF-kB complex in these cells. Based on these

observations, the present inventors assumed that specific downregulation of p65 expression in macrophages would affect intestinal inflammation. Thus, the functional role of p65 was investigated further using an antisense strategy.

Antisense experiments have been previously used to delineate various important functions of transcription factors. For instance, this strategy has revealed a central role for several DNA binding proteins in regulating cell growth and differentiation[27-33]. Now, the present inventors have surprisingly shown that lamina propria macrophages that were co-cultured with p65 antisense oligonucleotides produced strikingly lower levels of IL-1, IL-6 and TNF-a. This effect of the p65 antisense oligonucleotide was shown by previously established criteria to be both effective and specific[34-37]: (a) PCR and Western blot studies showed that the levels of p65 mRNA and protein were greatly reduced in cells exposed to the antisense oligonucleotide; (b) exposure of macrophages to the antisense oligonucleotide had no effect on a non-targeted protein (p50); (c) treatment of macrophages with several control oligonucleotides only minimally affected p65 levels; and (d) other unspecific effects, such as the CpG effect[26] or direct toxicity of the antisense oligonucleotide, were excluded.

Further in vivo studies demonstrated that TNBS-induced colitis can be successfully treated by systemic administration of p65 antisense oligonucleotides, even after the lesion is established. However, perhaps the most striking observation is that chronic TNBS-induced colitis can be successfully treated by a single local administration of the p65 antisense oligonucleotide. Moreover, by comparing different regimens of treatment in TNBS-induced colitis, local administration of p65 antisense oligonucleotides was found to be more effective in treating TNBS-induced colitis compared to local treatment with glucocorticoids. Furthermore, p65 antisense treatment appears to result in a longer-lasting and more profound improvement on clinical and histopathological parameters of colitis. In addition, no significant adverse effects of local p65 antisense treatment were observed raising the possibility that several of the problems with systemic antisense treatment reported can be avoided by the use of local application. However, these findings implicate local administration of antisense oligonucleotides as a novel strategy to treat chronic intestinal inflammation.

The above data suggest that the presence of p65 is essential to maintain TNBS-induced colitis and a persistent local activation of macrophages with concomitant cytokine

response. Therefore, the most likely mechanism by which p65 antisense oligonucleotides influence TNBS-induced colitis is the reduction of the local production of cytokines. This hypothesis is supported by the demonstration that macrophages from p65 antisense-treated mice produced strongly reduced levels of IL-1, IL-6 and TNF-a.

The central importance of the pluripotent cytokine TNF-a in chronic intestinal inflammation has been shown by the recent demonstration that Crohn's patients with established intestinal inflammation could be successfully treated by a single infusion of antibodies to TNF-a[38]. Similarly, the present inventors have recently found that antibodies to TNF-a partially reverse established TNBS-induced colitis in mice (M. F. Neurath, I. Fuss, W. Strober, K.-H. Meyer zum Büschenfelde, G. Kollias; unpublished data). Here, it was shown that antisense-induced p65 suppression was not only accompanied by reduced TNF-a levels but also by reduced production of several other proinflammatory cytokines by lamina propria macrophages. It appears therefore that specific local targeting of p65 in intestinal inflammation may permit downregulation of several different cytokine genes and thus might be more effective compared to antibody treatment for a single cytokine.

Intestinal microbial flora has been suggested to play an important role for the initiation and perpetuation of inflammatory bowel disease in humans[39]. Furthermore, it appears that the resident intestinal flora plays a major role in the pathogenesis of chronic intestinal inflammation found in TNBS-induced colitis[40] and the colitis found in IL-2-17, T cell receptor-20 and IL-10-18 deficient mice. In fact, it has been suggested that the primary defect in IL-10-deficient mice is a failure to control normal intestinal immune responses against enteric antigens leading to chronic inflammation via continuous overproduction of cytokines, such as TNF-a, IL-1 and IL-6. This hypothesis is supported by the finding that there is an almost 20-fold increase in the secretion of the proinflammatory cytokines IL-6 and TNF-a by LPS-stimulated spleen cells in IL-10 -/- mice compared to normal mice[18]. The deregulated activity of NF-kB p65 in IL-10 -/- mice suggests that one role of IL-10 in the normal gut may be to modulate the activity of NF-kB thereby indirectly affecting the expression levels of IL-1, IL-6 and TNF-a in macrophages[41]. Thus, the upregulation of these proinflammatory cytokines in the colitis in IL-10 -/- mice may be due to a deregulated activity of NF-kB p65 in the absence of IL-10 regulatory activity. Moreover, and by analogy, it is tempting to speculate that the colitis found in T cell receptor targeted mice could be explained by the absence of IL-10-producing Th2 cells

thereby abrogating a major and important suppressor of NF-kB DNA binding activity in those mice. However, the central role of NF-kB p65 in the IL-10 -/- model of chronic intestinal inflammation is further supported by the finding that chronic colitis in these mice can be successfully treated by p65 antisense oligonucleotides.

Subsequent studies in patients with inflammatory bowel disease revealed a similar upregulation of p65 expression by lamina propria macrophages in patients with Crohn´s disease. In addition, specific downregulation of p65 in these cells resulted in a considerably reduced production of IL-1, IL-6 and TNF-a. Surprisingly this effect on production of these cytokines appears to be more pronounced in comparison to treatment with 5-aminosalicylic acid or glucocorticosteroids. Thus, the usage of p65 antisense oligonucleotides is a highly effective way to treat chronic inflammation of the gut in humans. Furthermore, inhibition of NF-kB activity has been recently suggested as a major component of the anti-inflammatory activity of glucocorticoids [42-43]. Since glucocorticoids are frequently used for the treatment of chronic intestinal inflammation in humans[11-12], the present data imply a molecular explanation for the effect of local or systemic treatment with glucocorticoids in chronic colitis in humans.

The data reported here provide direct evidence for a predominant role of the p65 subunit of NF-kB in two murine models of chronic intestinal inflammation and the data strongly suggest that activation of p65 is essential to maintain chronic experimental colitis (Fig. 6). In addition, the present inventors have defined a novel way of delivering antisense oligonucleotides to the colon and to treat established colitis by p65 antisense oligonucleotides. Taken together with the functional importance of p65 for cytokine production in humans, the data presented here demonstrates the potential utility of local p65 antisense treatment in patients with Crohn´s disease (regional ileitis) and other inflammatory diseases of the gastrointestinal tract, such as colitis, ulcerative colitis, proctitis and colon irritabile. Additional examples of gastrointestinal diseases associated with increased cytokine production are e.g. celiac disease and colon irritabile. Local administration of the composition according to the present invention can further be of utility in mitigating the inflammatory symptoms in other diseases and inflammatory conditions of the gastrointestinal tract, such as e.g. gastric ulcers and duodenal ulcers.

In local treatment using the present oligonuclotide any suitable delivery mode or vehicle can be selected, provided that sufficient attention is paid to, on one hand preserv-

ing the stability and function of the oligonucleotide, and on the other hand to assuring, that the oligonucleotide is released in the right location, i.e. the area of inflammation. A preferable mode of administration in the treatment of conditions in the gastrointestinal tract is thus in the form of slow release compositions, such as coated tablets, capsules, suppositories and similar, conventionally used vehicles for delivery. Preferably the inventive oligonucleotide is included in a fat emulsion or, optionally, encapsulated in liposomes or other suitable carriers. The present oligonucleotide can also be administered included in aqueous solutions or gels, containing, in addition to the antisense oligonucleotide, suitable and pharmaceutically acceptable adjuvants, buffering agents and gelling agents. Suitable modes of administration of said solutions or gels can be gastric lavage, intestinal lavage, rectal lavage or administration of said solutions or gels orally or through sonds or catheters, either p.o. or per rectum. The precise composition of suitable pharmaceutical formulations can easily be determined by a person skilled in the art.

The novel, specific antisense oligonucleotide provides a novel molecular approach for the treatment of other related autoimmune inflammatory diseases, such as rheumatoid arthritis and localized neuritis, that are accompanied by increased local expression levels of IL-1, IL-6 and TNF-a. In the treatment of said diseases, the composition is preferably administered topically in the form of a salva, gel or ointment or by local intramuscular or sub dermal injections. Such compositions preferably also comprise suitable adjuvants, such as buffering agents, gelling agents, stabilizers and possible pigments.

Further examples of diseases and symptoms that could be treated in this manner includes e.g. psoriasis and hyperkeratosis, where the pathologic cell growth is associated with NF-kB expression. In the treatment of said diseases, the composition is preferably administered topically in the form of a salva, gel or ointment. Such compositions preferably also comprise suitable adjuvants, such as buffering agents, gelling agents, stabilizers and possible pigments.

The composition according to the present invention can naturally also be used in the treatment of other diseases that are accompanied by increased local expression levels, regardless of their cause, i.e. bacterial infections and macrophage triggered reactions. Different bacteria is often very opportune to colonize inflammated sites, in particular ulcers in the gastrointestinal tract and skin lesions associated with psoriasis, mentioning only two, non-limiting examples.

Examples

1. Methods

1.1 Animals

Specific pathogen-free 2-4 months old female SJL/J mice were obtained from the National Cancer Institute (NCI, Bethesda, MD). Animals were housed in the building 10A animal facility at the National Institutes of Health and maintained on standard laboratory chow and water ad libidum. IL-10 -/- mice have been previously described and characterized[18].

1. 2 Induction of TNBS-colitis

TNBS-colitis was induced as described[1]. In brief, the mice were lightly anaesthetized with metofane (methoxyflurane; Pitman-Moore, Mundelein, IL) after which 0.5 mg of 2,4,6-trinitrobenzene sulphonic acid (TNBS; obtained from Sigma, St. Louis, MO) in 50% ethanol or 50% ethanol alone was administered into the lumen of the colon via a 3.5F catheter fitted onto a 1 ml syringe. Animals were then kept in a vertical position for 30 seconds and returned to their cages.

1.3 Cell isolation and purification of lamina propria macrophages

Lamina propria mononuclear cells were isolated from freshly obtained colonic specimens. In brief, the resected colon was cut into 0.5 cm pieces and incubated twice in Ca- and Mg-free balanced salt solution containing EDTA (0.37 mg/ ml) and DTT (0.145 mg/ ml) in a shaking incubator at 37°C for 15 min to remove epithelial cells and intraepithelial lymphocytes. After decanting the supernatant the resultant colonic tissue was incubated for 90 min in RPMI-1640 containing 10% heat inactivated fetal calf serum, 25 mM Hepes, Collagenase D (400 U/ ml) (Boehringer Mannheim, Indianapolis, Indiana) and DNase I (0.1 mg/ml) (Boehringer Mannheim) in a shaking incubator at 37°C. The supernatant was collected by filtration straining through a 40 μm nylon cell strainer (Falcon, Becton Dickinson Labware, NJ). Lamina propria cells were then layered on a 40%-100% Percoll gradient (Pharmacia, Uppsala, Sweden) and centrifuged at 1500 rpm for 20 min at 4°C. Cells were then isolated at the 40-100% interface and further enriched for macrophages by negative selection technique using antibodies to CD4, CD8, B220, immunomagnetic beads and MACS technique (Miltenyi Biotec., Germany). As assessed by FACS analysis, the resultant cell population contained more than 85% F4/80 positive cells.

1.4. Isolation of primary splenic B cells

Primary splenic B cells were isolated as previously described[32]. The resultant cell population

contained more than 90% B220+ cells, as assessed by FACS analysis.Northern hybridization-Lamina propria mononuclear cells were isolated as described above and total cellular RNA from these cells was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method. 10 mg RNA was separated on agarose gels that were blotted onto 0.2 mm nitrocellulose membranes. Northern blots were hybridized to specific cDNA probes corresponding to IL-1, IL-6, TNF-a and β-actin sequences that were generated by RT-PCR amplification from splenic B cell cDNA.

1.5. Preparation of nuclear extracts

Small-scale extractions of nuclear proteins ("mini-extracts") were performed as previously described[44].

1.6 Electrophoretic mobility shift assays (EMSAs)

Gel retardation assays were performed using a DNA binding site for NF-kB in the TNF-a promoter[22]. Binding reactions (15 μl) for EMSA contained 2 μg synthetic DNA duplex of poly (dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ), 25000 cpm (Cerenkov) of end-labelled DNA probe for NF-kB, and incubation buffer (10 mM HEPES, pH 7.9, 100 mM NaCl, 10% glycerol, 0.5 mM MgCl2, 1 mM DTT). After preincubation without protein for 15 minutes at room temperature, crude nuclear proteins were added to the reaction for an additional 15 minutes and complexes were separated from unbound specific probe by electrophoresis in native 4% polyacrylamide gels. After electrophoresis, the gels were dried and exposed to Kodak films on intensifying screens overnight at 80°C.

1.7 Western blot analysis

For Western blotting, nuclear extracts from lamina propria macrophages were made at indicated time points as described above. 50 mg nuclear proteins from macrophages were blotted to a 0.45 mm nitrocellulose membrane followed by immunoblotting with a rabbit anti-p65 or p50 antibody (Santa Cruz Biotechnology, CA). After incubation with an AP-labelled goat anti-rabbit antibody detection was performed using AP color substrate (Promega) as previously described[44].

1.8 Shift Western blotting

The shift-Western blotting method was performed as described[45]. In brief, retarded protein-DNA complexes were transferred onto stacked nitrocellulose and anion-exchange membranes. The radiolabelled probe that bound to the anionic filter was detected directly, whereas the p65 protein was detected with p65 specific antibodies and ^{125}I-labelled protein A.

1. 9 Phosphorothioate oligonucleotides

Oligonucleotides were synthesized with a phosphorothioate backbone to improve resistance to endonucleases. Such phosphorothioate oligonucleotides were prepared by Genosys Biotechnologies, Inc. (Woodland, TX). The antisense oligonucleotide consisted of a 19-mer analogue to the 5´ end of the p65 subunit of NF-kB which spans the translation initiation site. In addition, two control (mismatched and non-sense) oligonucleotides were prepared. The non-sense oligonucleotide consisted of a 19-mer containing the same nucleotide composition of the anti-sense oligonucleotide. The sequences of murine and human phosphorothioate oligonucleotides were as follows:

human p65 antisense: 5´-GGAACAGTTCGTCCATGGC-3´
human p65 mismatched: 5´-GGAACAGTTCGTCTATGGC-3´
human p65 non-sense: 5´-TACAGAGGTGCTCACTGGC-3´
murine p65 antisense: 5´-GAAACAGATCGTCCATGGT-3´
murine p65 mismatched: 5´-GAAACAGATCGTCTATGGT-3´
murine p65 non-sense: 5´-GTACTACTCTGAGCAAGGA-3´

1.10 Cell culture of lamina propria macrophages

Cell cultures of lamina propria macrophages were performed in complete medium consisting of RPMI-1640 supplemented with 3 mM L-glutamine, 10 mM HEPES buffer, 10 μg/m gentamycin (Whittaker), 100 U/ m each of penicillin and streptomycin (Whittaker), 0.05 mM 2ME (Sigma Chemical) and 10% heat-inactivated fetal calf serum. Co-incubation studies with phosphorothioate oligonucleotides were performed in 24 well plates with indicated concentrations of oligonucleotides.

1.11 Proliferation assays

Proliferation of primary splenic B cells was assessed by measuring [3H]-TdR incorporation during the final eight hours of cell culture. In brief, 5 x 104 cells/ m were cultured in flat-bottomed 96-well plates (Costar Corp.) for 24 hours; during the last 8 hours of culture, 1 μCi of [3H]-TdR (New England Nuclear, Boston, MA) (specific activity 6.7 Ci/ mmol) was added to each well; incorporated [3H] radioactivity was measured in a scintillation counter (LS2800; Beckman Instruments, Inc., Fullerton, CA). Each incubation experiment was done in triplicate.

1.12 Reagents and Monoclonal Antibodies

Unconjugated and biotinylated monoclonal rat anti-mouse IL-1, IL-6, and TNF-a antibodies

and recombinant mouse cytokines were purchased from Pharmingen (San Diego, CA) and Genzyme Corp. (Cambridge, MA) respectively. Human cytokines and antibodies to human cytokines were obtained from R & D Systems (Minneapolis, MN).

1.13 Cytokine Assays

To measure cytokine production, cultures were incubated in 24-well plates (Costar) at 37°C in a humidified incubator containing 6% CO_2. At indicated time points, culture supernatants were removed and assayed for cytokine concentration. Cytokine concentrations were determined by specific ELISA according to the manufacturer's recommendation. Optical densities were measured on a Dynatech MR 5000 ELISA reader at a wavelength of 490 nm.

1.14 In vivo administration of anti-sense oligonucleotides

Animals were injected intravenously or intrarectally at indicated time points after administration of TNBS. Weight changes were monitored as described[1] and organs were taken and analyzed at indicated time points.

1.15 Histologic analysis

Tissues were removed from TNBS-treated mice at indicated time points and fixed in 10% buffered neutral formaldehyde for paraffin embedding. Paraffin sections were made and stained with haematoxylin and eosin.

1.15 Patients

Colonic specimens obtained from 41 surgical patients admitted for bowel resection were studied. The Crohn´s disease group consisted of 9 men and 9 women, ranging from 23 to 61 years of age. At the time of resection, 5 patients were receiving corticosteroids, 7 patients were receiving an oral sulfasalazine preparation and 6 patients were on no medications. The control group consisted of 23 patients admitted for therapeutic bowel resection for malignant (n=13) and nonmalignant (n=10) conditions, ranging from 17 to 75 years of age. These patients were not receiving any medications at the time of the resection.

1.16 Isolation of human lamina propria macrophages

Lamina propria mononuclear cells were isolated using a previously described technique[46]. Lamina propria macrophages were prepared from the resultant cell population by a negative selection technique using monoclonal antibodies attached to immunomagnetic beads. In brief, cell populations were suspended at 2 x 107 cells/m in calcium free PBS with 1% FCS to which a 1:350 dilution of ascites fluid containing the antibodies OKT8, OKT4 and anti-erythroglycoprotein was added. The cells were incubated at 4°C for 30 min, washed twice and

resuspended in coating medium. The antibody coated cell populations were then removed by an initial incubation with immunomagnetic beads coated with anti-murine IgG antibody (Advanced Magnetics, Cambridge MA) followed by a subsequent incubation with immunomagnetic beads coated with anti-murine IgG antibody obtained from Dynal (Oslo, Norway). The resultant cells were resuspended in complete RPMI media and cultured in complete media with or without 40 mg/m LPS and 10 mg/m phytohaemagglutinin (PHA; both obtained from Sigma Chem., St. Louis). To some of the samples, corticosteroids, 5-aminosalicylic acid or phosphorothioate oligonucleotides were added at a final concentration of 8 mM.

1.17 Statistic analysis

Tests for significance of differences were made by student's t-test using the program StatWorks.

2. Examples

Example 1: Upregulation of p65 expression in chronic intestinal inflammation

The present inventors have recently described a novel murine model of chronic intestinal inflammation induced by the haptenizing reagent 2,4,6-trinitrobenzene sulphonic acid (TNBS) that mimics some important characteristics of Crohn's disease in humans[1]. In particular, TNBS-induced colitis is dominated by CD4+ T cells producing Th1-type cytokines and macrophages. The latter cell population is known to produce several important proinflammatory cytokines and has been linked to the pathogenesis of inflammatory bowel disease in humans[11-12]. To further characterize the role of local intestinal macrophages in the TNBS colitis model, the present inventors assayed the ability of lamina propria macrophages from TNBS-treated mice to produce various proinflammatory cytokines. As shown in Figure 1a, there was a strikingly increased production of IL-1, IL-6 and TNF-a mRNAs by macrophages in TNBS-induced colitis compared with those in normal mice. This finding was consistent with an increased secretion of these cytokines, as assessed by ELISA studies (Table 1), consistent with previous reports on cytokine profiles in patients with Crohn's disease[11-12].

Table 1 - Cytokine production by murine lamina propria macrophages

	IL-1 (pg/ml)	IL-6 (pg/ml)	TNF-α (U/ml)
normal	4 +/- 0.3	23 +/- 3.5	24 +/- 5.5
ethanol	4 +/- 0.5	20 +/- 1.7	35 +/- 7.1
TNBS/ ethanol	54 +/- 7.4	195 +/- 16.8	387 +/- 49.2
p65 antisense	12 +/- 3.9	9 +/- 4.1	11 +/- 3.1
p65 non-sense	57 +/- 8.8	178 +/- 11.9	369 +/- 44.1
p65 mismatched	61 +/- 7.7	191 +/- 20.5	394 +/- 33.7

(Secretion of IL-1, IL-6 and TNF-a by lamina propria macrophages from normal mice, ethanol- or TNBS-treated mice and from mice with TNBS-induced colitis treated with phosphorothioate oligonucleotides at day 21. Lamina propria macrophages were isolated from normal SJL/J mice and from untreated and treated mice with TNBS-induced colitis at day 21 after administration of TNBS and cultured in complete medium. Supernatants were collected after 24 hours and analyzed for cytokine content by specific ELISA. One representative experiment out of three is shown. Standard errors are indicated.)

One major control mechanism of gene expression occurs at the transcriptional level and all of these proinflammatory cytokines have been shown to be regulated by the transcription factor NF-kB via binding of NF-kB family members to their individual promoters[6, 22-23]. To investigate whether the activity of different NF-kB family members was altered in TNBS-induced colitis, nuclear proteins from lamina propria macrophages in TNBS-induced colitis were extracted and analyzed by gel retardation assays.

A striking increase of NF-kB DNA binding activity in nuclear extracts from these cells was observed (Fig. 1b). Subsequent Western and shift-Western blotting experiments identified the p50 (data not shown) and p65 subunits of NF-kB as major components in this retarded protein complex (Fig. 1c). Whereas p50 is a poor transcriptional activator, the p65 subunit of NF-kB has been shown to mediate transcriptional activation of a cascade of genes linked to the control of cell proliferation and differentiation upon mitogenic stimulation[24-25]. Based on these observations, the present inventors focused in further studies on the functional role of p65 in chronic intestinal inflammation.

Example 2: An antisense oligonucleotide to the translation start site of p65 specifically down-regulates IL-1, IL-6 and TNF-a production by lamina propria macrophages

To directly test whether a specific antisense oligonucleotide targeting the translation initiation site of murine p65 would affect the expression level of p65, lamina propria macrophages from mice with TNBS-induced colitis were co-incubated in cell culture with phosphorothioate oligonucleotides. The p65 antisense oligonucleotide strikingly reduced the expression of p65 at both the mRNA (data not shown) and protein levels (Fig. 2). This inhibition was specific for p65 since the expression level of another unrelated gene (p50) was not affected in the same nuclear extracts. Additional viability studies showed no direct toxic effects of the p65 antisense oligonucleotides (Fig. 2). No downregulation of p65 was observed when mismatched or "non-sense" control oligonucleotides were used. Furthermore, the antisense-induced downregulation of p65 expression was accompanied by reduced secretion of IL-1 (4.5-fold), IL-6 (21-fold) and TNF-a (35-fold) by LPS-stimulated macrophages, as assessed by ELISA (table 1), further supporting the idea that p65 is a major transcriptional regulator controlling the expression of these cytokines.

Example 3: In vivo administration of p65 antisense oligonucleotides abrogates established experimental colitis

Having demonstrated the ability of the antisense oligonucleotide to downregulate p65 expression and subsequent cytokine production by lamina propria macrophages, the present inventors next assessed the functional role of p65 in chronic TNBS-induced colitis in vivo. Phosphorothionate oligonucleotides were administered to mice with chronic TNBS-induced colitis either as a single intravenous injection or applied locally into the colon by injection via a catheter. It was found that a single intravenous injection of p65 antisense oligonucleotides abrogated clinical signs of established intestinal inflammation. Antisense-treated mice did no longer have diarrhoea and started to gain weight (Fig. 3a). In contrast, no significant clinical changes were observed in mice treated with control oligonucleotides.

Perhaps even more strikingly, it was found that TNBS-induced colitis could be successfully treated by a single local administration of the p65 antisense oligonucleotide (Fig. 3a). There was no apparent clinical sign of toxicity of the p65 antisense oligonucleotide at the concentrations tested (150-1000 mg/ mouse). In addition, no significant changes in the serum levels of AST (66+/-1 U/l vs. 59+/-14 U/l), ALT (31+/-8 U/l vs. 24+/-7U/l), glucose (122+/-12 mg/dl vs. 127+/-10 mg/dl) and creatinine (0.5+/-0.04 mg/dl vs. 0.5+/-0.06

mg/dl) were found between p65 antisense-treated and untreated mice. No increase in the proliferation rate (110860+/-17110 vs. 198980 +/- 20150 CPM) of LPS-activated primary splenic B cells was found in p65 antisense-treated mice compared to untreated mice excluding an unspecific stimulating effect of CpG motifs in the antisense oligonucleotide on B cell proliferation[26]. Furthermore, there were no histopathologic alterations in the spleen, thymus, pancreas, brain, liver and kidney of p65 antisense-treated mice (data not shown).

Histopathological studies of the colon of p65 antisense-treated mice showed a complete abrogation of intestinal inflammation 7 days after administration of oligonucleotides (Figs. 3b-d). Moreover, macrophages obtained from the lamina propria of p65 antisense-treated mice produced significantly lower amounts of IL-1, IL-6 and TNF-a mRNA in cell culture (Fig. 3e).

Example 4: A single local administration of p65 antisense oligonucleotides is more effective in treating TNBS-induced colitis than local administration of glucocorticoids

In further studies, the present inventors compared the effects of glucocorticoids and p65 antisense oligonucleotides on the clinical course of chronic TNBS-induced colitis. When mice with established TNBS-induced colitis were treated by a single local administration of glucocorticoids, only a small short-lasting increase in the average body weight was observed (Fig. 4a). However, daily local administration of glucocorticoids was more effective in treating chronic colitis but mice still showed evidence of colonic inflammation, as assessed by histologic analysis (data not shown) and weight curves (Fig. 4a). Daily systemic administration of glucocorticoids was found to be more effective than daily local treatment but less effective than administration of p65 antisense oligonucleotides (Fig. 4b).

Example 5: Predominant role of p65 in chronic intestinal inflammation in IL-10-/- mice

Since the above data suggested a predominant role for the p65 subunit of NF-kB in chronic TNBS-induced colitis, the present inventors set out to determine the role of p65 in yet another murine model of chronic intestinal inflammation. Here, the present inventors chose to analyze the function of NF-kB p65 in the IL-10 -/- model of chronic intestinal inflammation that is characterized by infiltrates of granulocytes, lymphocytes and macrophages in the gut[18]. It was found that lamina propria macrophages in these mice displayed strikingly higher levels of NF-kB binding activity and Western blot studies showed an increase of p65 expression by macrophages in these mice compared to wild-type littermates (Fig. 5). When IL-10 -/- mice with chronic intestinal inflammation were treated with p65 antisense

oligonucleotides, the present inventors found a surprising abrogation of wasting disease with a reduction of the macroscopic and histologic signs of inflammatory activity (data not shown) suggesting that p65 is essential in this model to maintain chronic intestinal inflammation.

Example 6: Increased production of NF-kB p65 by lamina propria macrophages in patients with Crohn´s disease

Based on the predominant role for NF-kB p65 in two murine models of chronic intestinal inflammation, the present inventors focused in further studies on the question whether a deregulated activity of p65 is found in patients with Crohn´s disease in humans. Accordingly, the present inventors purified lamina propria macrophages from patients with Crohn´s disease using negative selection techniques and analyzed the expression of NF-kB p65 in stimulated and unstimulated cells by Western and shift-Western blot analysis. As assessed by densitometry, a significant upregulation of p65 levels was found in patients with Crohn´s disease ($p < 0.01$): There was on average a 14.2-fold increase of p65 expression in unstimulated and a 36.5-fold increase in LPS-stimulated lamina propria macrophages in patients with Crohn´s disease ($n=18$) compared to macrophages obtained from control specimens ($n=23$; see Methods) suggesting a continuous activation of NF-kB p65 in patients with this disease. When lamina propria macrophages from patients with Crohn's disease were co-cultured with p65 antisense oligonucleotides, a strongly reduced production of IL-1, IL-6 and TNF-a was found (table 2). No such effect was observed when lamina propria macrophages were co-incubated with control non-sense or mismatched oligonucleotides. The downregulatory effect of the p65 antisense oligonucleotide on cytokine production by macrophages was more pronounced than the ones observed with 5-aminosalicylic acid or glucocorticoids. These data support the finding that p65 is a key factor in deregulating the expression of proinflammatory cytokines, such as IL-1, IL-6 and TNF-a, in Crohn disease and suggest that the usage of p65 antisense oligonucleotides may be a highly effective way to treat chronic inflammation of the gut in humans.

Table 2 - Cytokine production by lamina propria macrophages in Crohn's disease[a]

	IL-1 (pg/ml)		IL-6 (pg/ml)		TNF-α (pg/ml)	
group	media	LPS PHA	media	LPS PHA	media	LPS PHA
control patients (n= 23)	20	43	23	108	10	323
Crohn´s patients (n= 18)	265	455	1777	3845	134	1878

Table 2 continued

Crohn's patients (n= 18)	IL-1 (pg/ml) media	IL-1 (pg/ml) LPS PHA	IL-6 (pg/ml) media	IL-6 (pg/ml) LPS PHA	TNF-α (pg/ml) media	TNF-α (pg/ml) LPS PHA
untreated	265	455	1777	3845	134	1878
5-aminosalicylic acid	138	191	842	1321	85	388
corticosteroids	78	121	647	711	45	184
p65 antisense	66	109	132	218	12	44
p65 non-sense	288	412	1912	3982	211	1721
p65 mismatched	204	423	1657	3321	201	1903

("Secretion of IL-1, IL-6 and TNF-a by lamina propria macrophages from patients with Crohn's disease and control patients. Lamina propria macrophages were isolated from bowel specimens and cultured in complete media with or without 40 mg/m LPS and 10 mg/m phytohaemagglutinin (PHA) in the presence or absence of corticosteroids, 5-aminosalicylic acid or phosphorothioate oligonucleotides. Supernatants were collected after 48 hours and analyzed for cytokine content by specific ELISA.)

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

References

1. Neurath, M. F., Fuss, I., Kelsall, B. L., Stuber, E. & Strober, W. Antibodies to IL-12 abrogate established experimental colitis in mice. J. Exp. Med. 182, 1280-1289 (1995).

2. Stüber, E., Strober, W. & Neurath, M. F. Blocking the CD40L-CD40 interaction in vivo specifically prevents the priming of Th1-T cells through the inhibition of IL-12 secretion. J. Exp. Med. 183, 693-698 (1996).

3. Cross, S. L. et al. Functionally distinct NF-kB binding sites in the Ig kappa and IL-2 receptor alpha chain genes. Science 244, 466-469 (1989).

4. Finco, T. S. & Baldwin, A. S. Mechanistic aspects of NF-kB regulation: the emerging role of phosphorylation and proteolysis. Immunity 3, 263-272 (1995).

5. Lenardo, M. J. & Baltimore, D. NF-kB: a pleiotropic activator of inducible and tissue specific gene control. Cell 58, 227-237 (1989).

6. Baeuerle, P. A. & Henkel, T. Function and activation of NF-kB in the immune system. Ann. Rev. Immunol. 12: 141-179 (1994).

7. Sha, W. et al. Targeted disruption of the p50 subunit of NF-kB leads to multifocal defects in immune responses. Cell 80, 321-330 (1995).

8. Weih, F. et al. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kB/Rel family. Cell 80, 331-340 (1995).

9. Köntgen, F. et al. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes Dev. 9, 1965-1977 (1995).

10. Burkly, L. et al. Expression of relB is required for the development of thymic

medulla and dendritic cells. Nature 373, 531-536 (1995).

11. Podolsky, D.K. 1991. Inflammatory bowel disease. New Engl. J. Med. 325, 928-935 (1995).

12. Strober, W. & Neurath, M. F. Immunological diseases of the gastrointestinal tract. in: R. R. Rich (ed) Clinical Immunology, Chapter 94. Mosby, St. Louis. 1401-1428 (1995).

13. Strober, W. & Ehrhardt, R. O. Chronic intestinal inflammation: an unexpected outcome on cytokine or T cell receptor mutant mice. Cell 75, 203-205 (1993).

14. Hammer, R. E. et al. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human β2m: an animal model of HLAB-2-associated human disorders. Cell 63, 1099-1112 (1990).

15. Hermiston, M. L. & Gordon, J. I. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. Science 270, 1203-1207 (1995).

16. Holländer, G. A. et al. Severe colitis in mice with aberrant thymic selection. Immunity 3, 27-38 (1995).

17. Sadlack, B. et al. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75, 253-261 (1993).

18. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. & Müller, W. Interleukin-10--deficient mice develop chronic enterocolitis. Cell 75, 263-274 (1993).

19. Rudolph, U. et al. Ulcerative colitis and adenocarcinoma of the colon in Gai2--deficient mice. Nature Genetics 10, 143-146 (1995).

20. Mombaerts, P. et al. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. Cell 75, 275-282 (1993).

21. Powrie, F. et al. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity 1, 553-562 (1994).

22. Shakhov, A. N. et al. kB-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor a gene in primary macrophages. J. Exp. Med. 171, 35-47 (1990).

23. Libermann, T. A. & Baltimore, D. Activation of interleukin-6 gene expression through the NF-kB transcription factor. Mol. Cell. Biol. 10, 2327-2334 (1990).

24. Higgins, K. A. et al. Antisense inhibition of the p65 subunit of NF-kB blocks tumorigenicity and causes tumor regression. Proc. Natl. Acad. Sci. USA 90, 9901-9905 (1993).

25. Kitajima, I, Shinohara, T, Bilakovics, J et al. Ablation of transplanted HTLV-I Tax-transformed tumors in mice by antisense inhibition of NF-kB. Science 258, 1792-1795 (1992).

26. Krieg, A. M. CpG motifs in bacterial DNA trigger direct B cell activation. Nature 374, 546-549 (1995).

27. Loke, S. L. et al. Delivery of c-myc antisense phosphorothioate oligodeoxynucleotides to hematapoietic cells in culture by liposome fusion: specific reduction in c-myc protein expression correlates with inhibition of cell growth and DNA synthesis. Curr. Top. Microbiol. Immunol. 141, 282-288 (1988).

28. Venturelli, D., Travali, S. & Calabretta, B. Inhibition of T-cell proliferation of a myb antisense oligomer is accompanied by selective down-regulation of DNA polymerase alpha expression. Proc. Natl. Acad. Sci. USA 87, 5963-5967 (1990).

29. Voltieri, M. et al. Antisense myb inhibition of purified erythroid rogenitors in development and differentiation is linked to cyclin activity and expression of DNA

polymerase alpha. Blood 77, 1181-1190 (1991).

30. Holt, J. T. et al. Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation. Proc. Natl. Acad. Sci. USA 83, 4794-4798 (1986).

31. Nishikura, K. & Murray, J. M. Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells. Mol. Cell. Biol. 7, 639-648 (1987).

32. Wakatsuki, Y., Neurath, M. F., Max, E. E. & Strober, W. The B cell specific transcription factor BSAP regulates B cell proliferation. J. Exp. Med. 179, 1099-1108 (1994).

33. Gura, T. Antisense has growing pains.Science 270: 575-577 (1995).

34. Wagner, R. W. et al. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. Science 260, 1510-1514 (1993).

35. Wagner, R. W. Gene inhibition using antisense oligodeoxynucleotides. Nature 372, 333-335 (1994).

36. Nesterova, M. & Cho-Chung Y. S. A single-injection protein kinase A-directed antisense treatment to inhibit tumour growth. Nature Med. 1, 528-533 (1995).

37. Holt, JT. A 'senseless' immune response to DNA. Nature Med. 1, 407-408 (1995).

38. van Dullemen, H. M. et al. Treatment of Crohn´s disease with anti-tumour necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 109, 129-135 (1995).

39. Sartor, R. B. Role of intestinal microflora in initiation and perpetuation of inflammatory bowel disease. Can. J. Gastroenterol. 4, 271-277 (1990).

40. Duchmann, R., Schmitt, E., Knolle, P., Meyer zum Büschenfelde, K.-H. & Neurath,

M. F. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. Eur. J. Immunol.; in press (1996).

41. Wang, P., Wu, P., Siegel, M. I., Egan, R. W. & Billah, M. M. Interleukin (IL)-10 inhibits nuclear factor B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J. Biol. Chem. 270, 9558-9563 (1995).

42. Scheinman, R. I., Cogswell, P. C., Lofquist, A. K. & Baldwin, A. S. Role of transcriptional activation of IkBa in mediation of immunosuppression by glucocorticoids.-Science 270, 283-286 (1995).

43. Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. & Karin, M. Immunosuppression by glucocorticoids: inhibition of NF-kB activity through induction of IkBa synthesis.Science 270, 286-290 (1995).

44. Neurath, M. F., Max E. E. & Strober W. Pax5 (BSAP) regulates the murine immunoglobulin 3'a enhancer by affecting binding of NF-aP, a protein that controls heavy chain transcription. Proc. Natl. Acad. Sci. USA 92, 5336-5340 (1995).

45. Demczuk, S., Harbers, M. & Vennström, B. Identification and analysis of all components of a gel retardation assay by combination with immunoblotting. Proc. Natl. Acad. Sci. USA 90, 2574-2578 (1993).

46. Bull, D. M. & Bookman, M. A. Isolation and functional characterization of human intestinal mucosal mononuclear cells. J. Clin. Invest. 59, 966-974 (1977).

Claims

1. Pharmaceutical composition for treatment of inflammatory diseases, in particular for the downregulation of cytokine expression, **characterized** in that said composition contains a therapeutically effective amount of p65 antisense oligonucleotide.

2. Pharmaceutical composition according to claim 1, **characterized** in that said p65 antisense oligonucleotide contains the following nucleotide sequence:

5´-GGAACAGTTCGTCCATGGC-3´

or homologues thereof.

3. Method in the diagnosis of inflammatory conditions in humans, **characterized** in that the level of p65 expression in a sample of macrophages taken in a specific locus in the patient is determined in vitro and a found level is compared to the level for healthy individuals or the level obtained at another occasion for the same individual.

4. Method according to claim 3, **characterized** in that the level of p65 expression is determined in a sample of lamina propria macrophages taken in the intestines of a patient.

5. Method according to claim 3, **characterized** in that the level of p65 expression is determined in a sample of macrophages taken in a fluid biopsy of a patient.

6. Method according to claim 4, **characterized** in that an increased level of p65 expression is taken as a sign of an inflammatory gastrointestinal disease.

7. Method according to claim 5, **characterized** in that an increased level of p65 expression is taken as a sign of rheumatoid arthritis.

8. Method according to any one of claims 3 to 7, **characterized** in that said p65 antisense oligonucleotide contains the following nucleotide sequence:

5´-GGAACAGTTCGTCCATGGC-3´

or homologues thereof.

9. Method for the treatment of inflammatory conditions in humans, **characterized** in that a therapeutically effective amount of p65 antisense oligonucleotide is administered locally.

10. Method according to claim 9, **characterized** in that said p65 antisense oligonucleotide contains the following nucleotide sequence:

5´-GGAACAGTTCGTCCATGGC-3´

or homologues thereof.

11. Method according to claim 10, **characterized** in that a therapeutically effective amount of p65 antisense oligonucleotide is administered locally to the intestines of a patient.

12. Method according to claim 10, **characterized** in that a therapeutically effective amount of p65 antisense oligonucleotide is administered locally to the synovial areas of a patient.

13. Method according to claim 10, **characterized** in that a therapeutically effective amount of p65 antisense oligonucleotide is administered topically to the skin of a patient.

14. Method according to any one of claims 11 to 12, **characterized** in that the therapeutically effective amount of p65 antisense oligonucleotide is administered locally incorporated in any one of the following combinations: an aqueous solution, a fat emulsion, coated tablets, capsules, suppositories, microcapsules or liposomes.

15. Method according to any one of claims 12 to 13, **characterized** in that the therapeutically effective amount of p65 antisense oligonucleotide is administered locally incorporated in any one of the following combinations: an aqueous gel, a fat emulsion, microcapsules or liposomes.

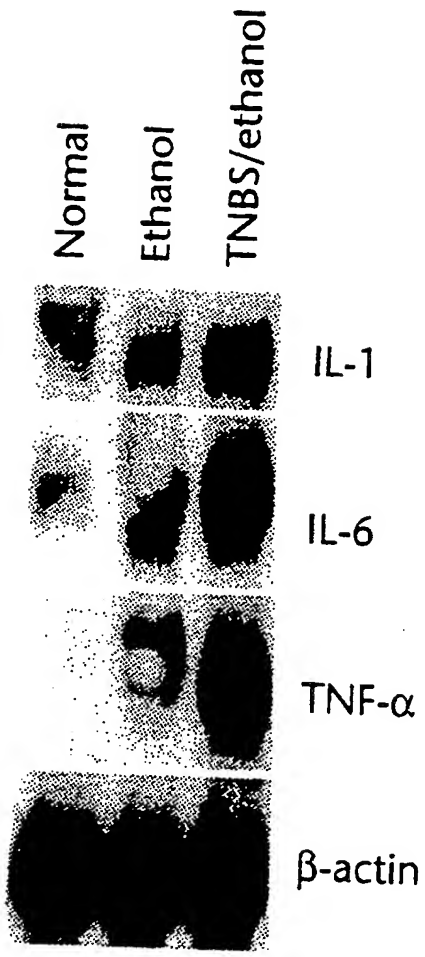
16. Use of a p65 antisense oligonucleotide for the manufacture of a pharmaceutical composition for local administration.

17. Use of a p65 antisense oligonucleotide for the manufacture of reagents, useful for the determination of the level of p65 expression in a sample.

18. Use according to any one of claim 16-17, **characterized** in that said p65 antisense oligonucleotide contains the following nucleotide sequence:

5´-GGAACAGTTCGTCCATGGC-3´

or homologues thereof.



NORTHERN

Fig. 1a

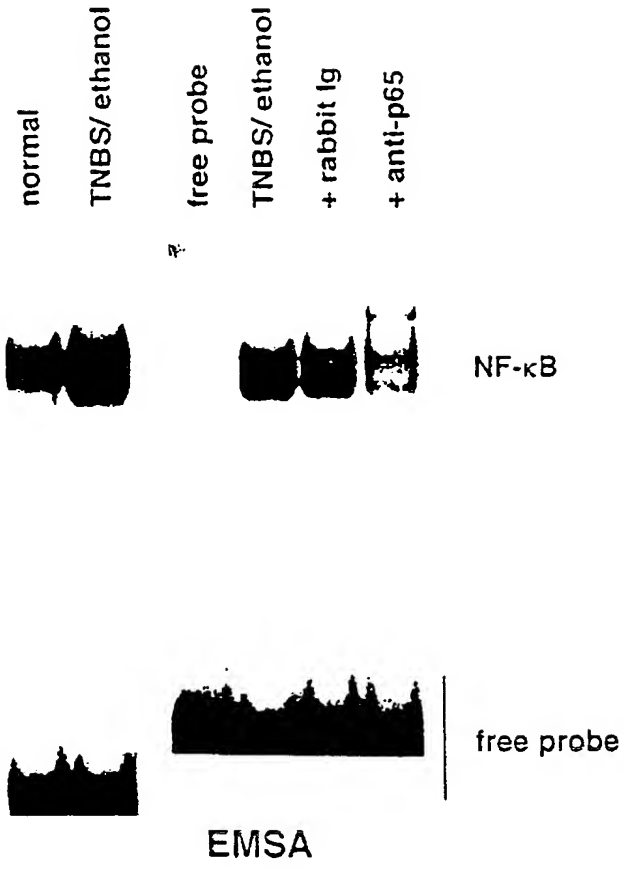

normal
TNBS/ ethanol
free probe
TNBS/ ethanol
+ rabbit Ig
+ anti-p65
NF-κB
free probe
EMSA

Fig. 1b

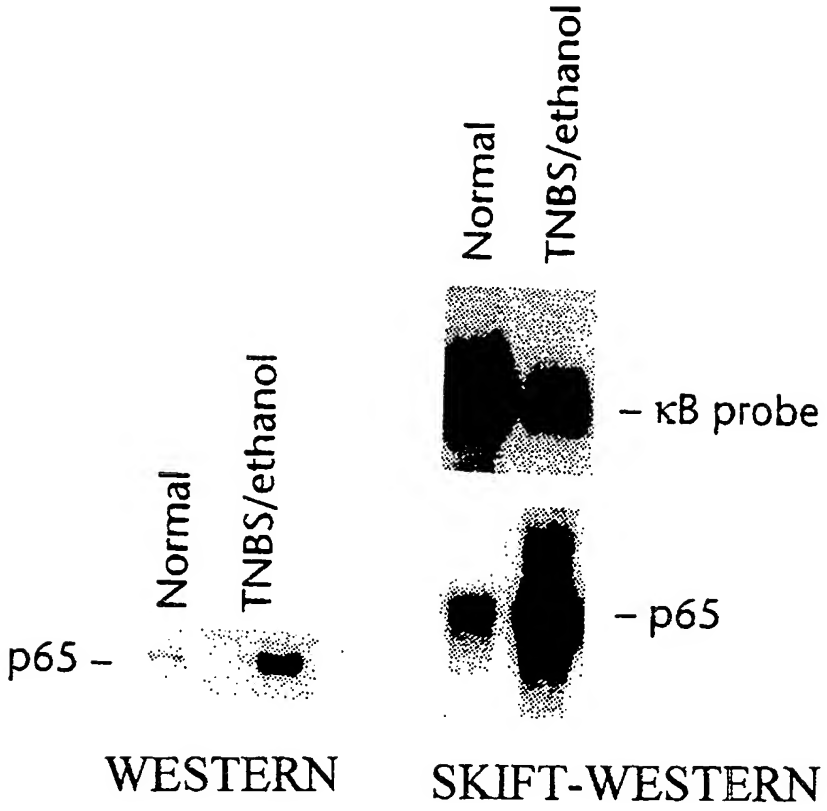
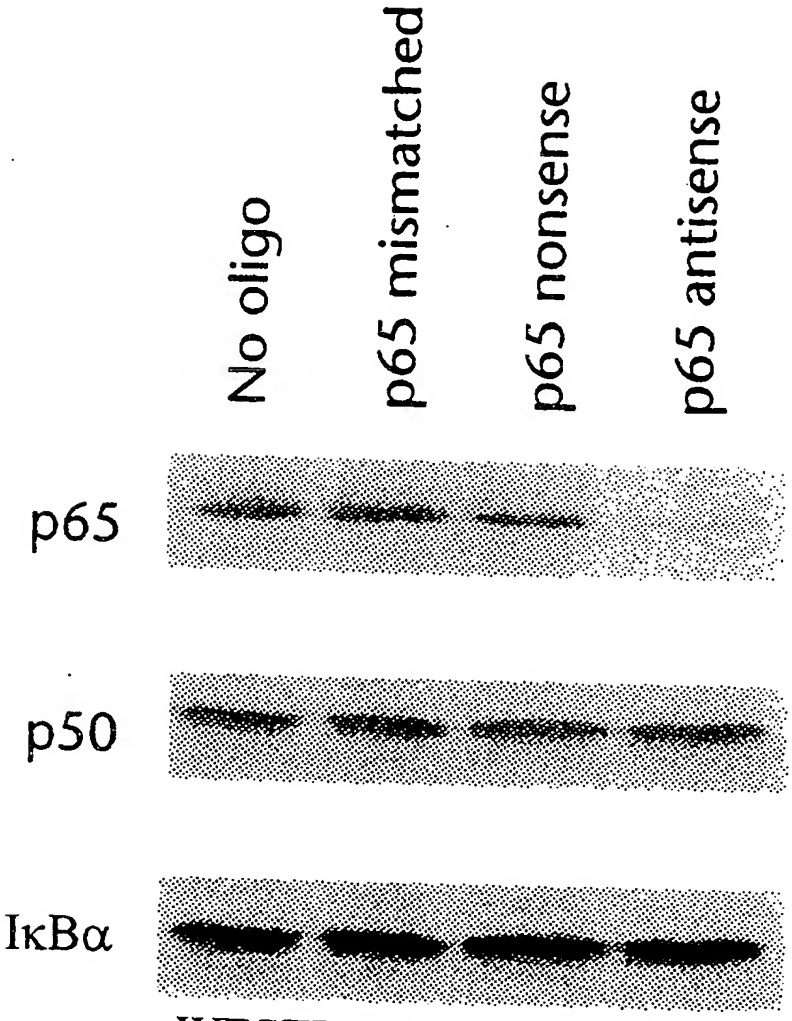

Normal
TNBS/ethanol
– κB probe
– p65
Normal
TNBS/ethanol
p65 –
WESTERN
SKIFT-WESTERN

Fig. 1c



WESTERN

Fig. 2

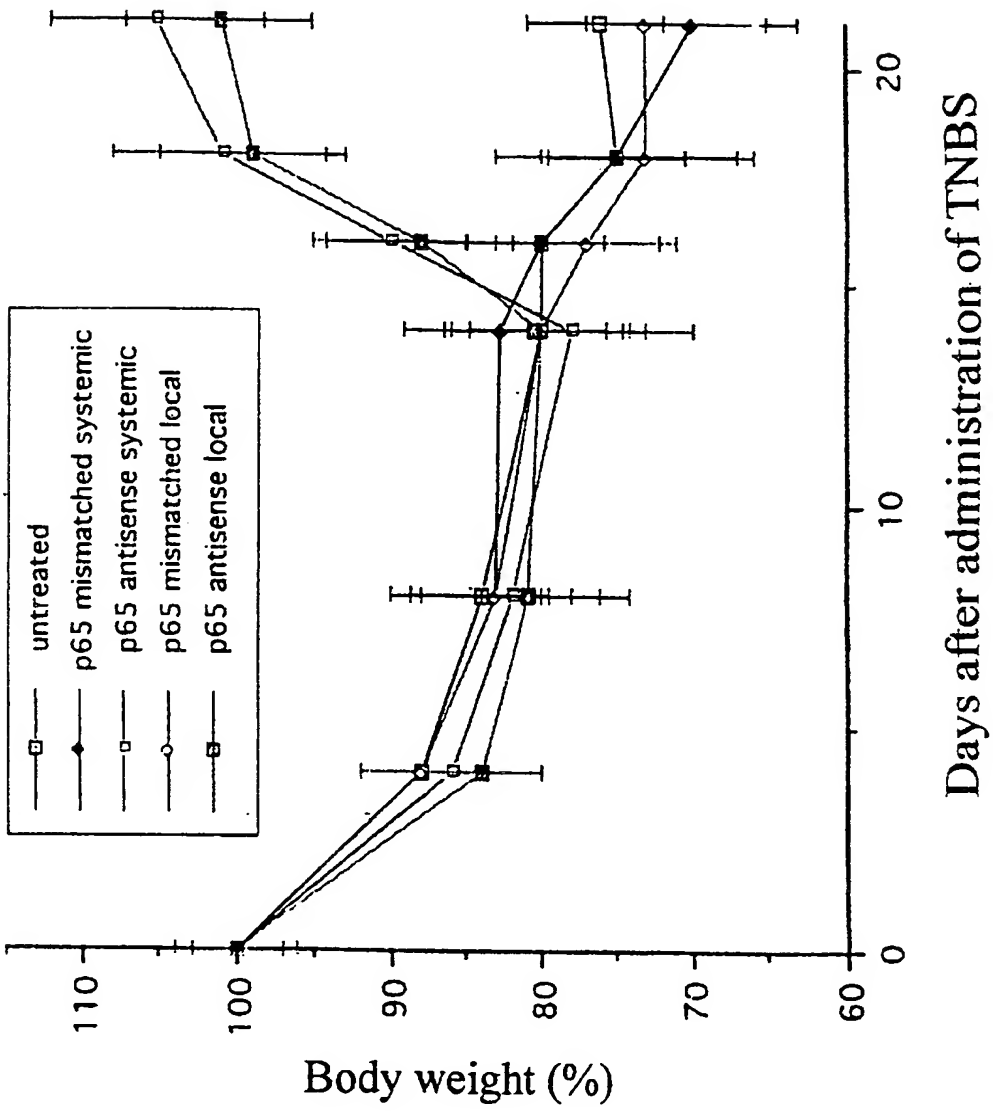

untreated
p65 mismatched systemic
p65 antisense systemic
p65 mismatched local
p65 antisense local
110
100
90
80
70
60
0
10
20
Body weight (%)
Days after administration of TNBS

Fig. 3a

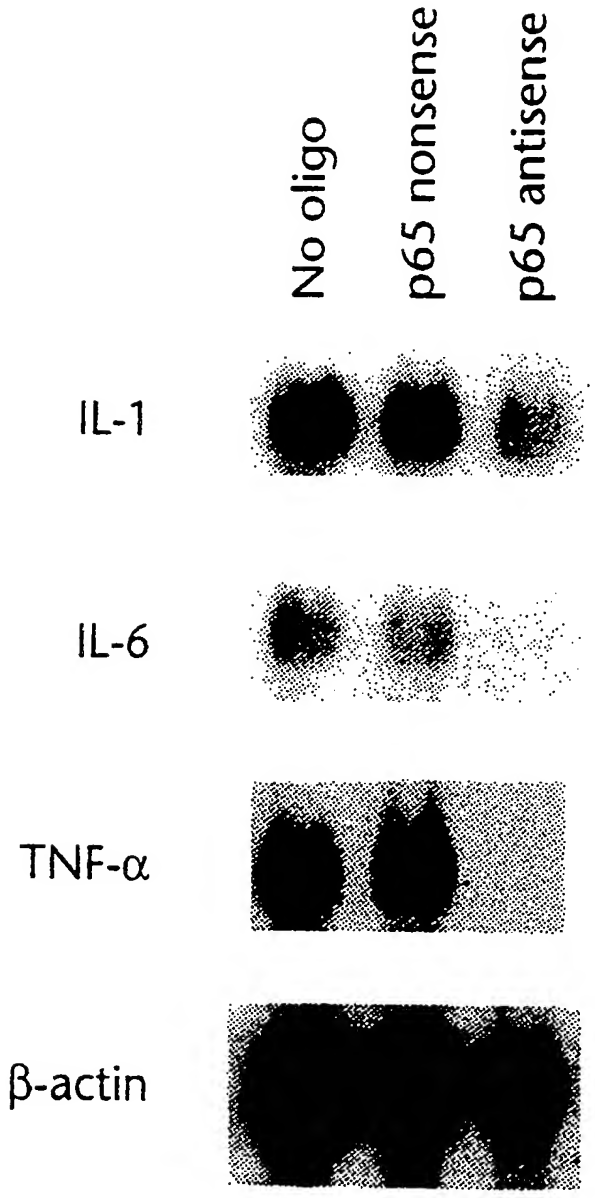

No oligo
p65 nonsense
p65 antisense
IL-1
IL-6
TNF-α
β-actin
NORTHERN

Fig. 3b

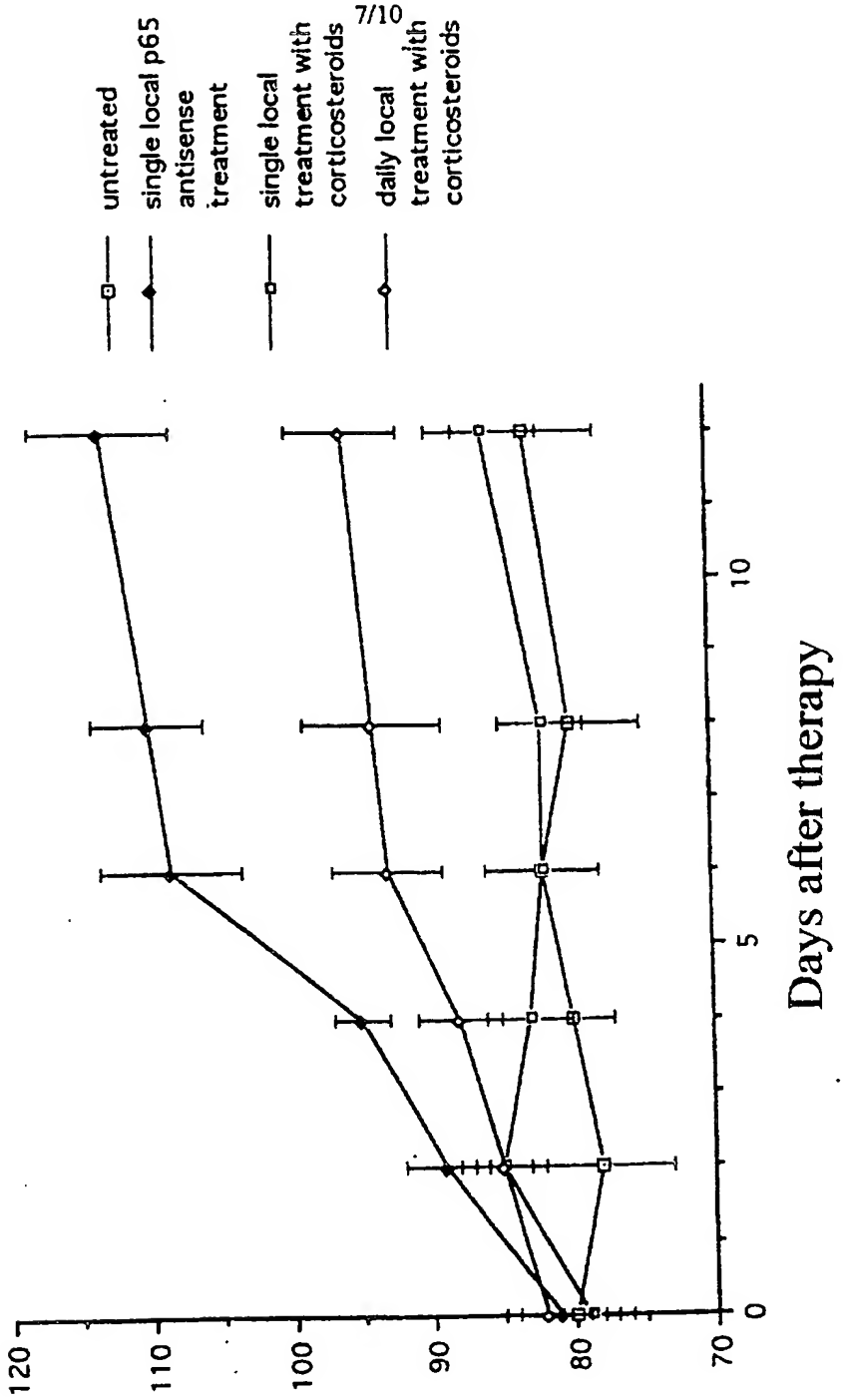

untreated
single local p65 antisense treatment
single local treatment with corticosteroids
daily local treatment with corticosteroids
120
110
100
90
80
70
0
5
10
Days after therapy
Percentage of initial body weigth

Fig. 4a

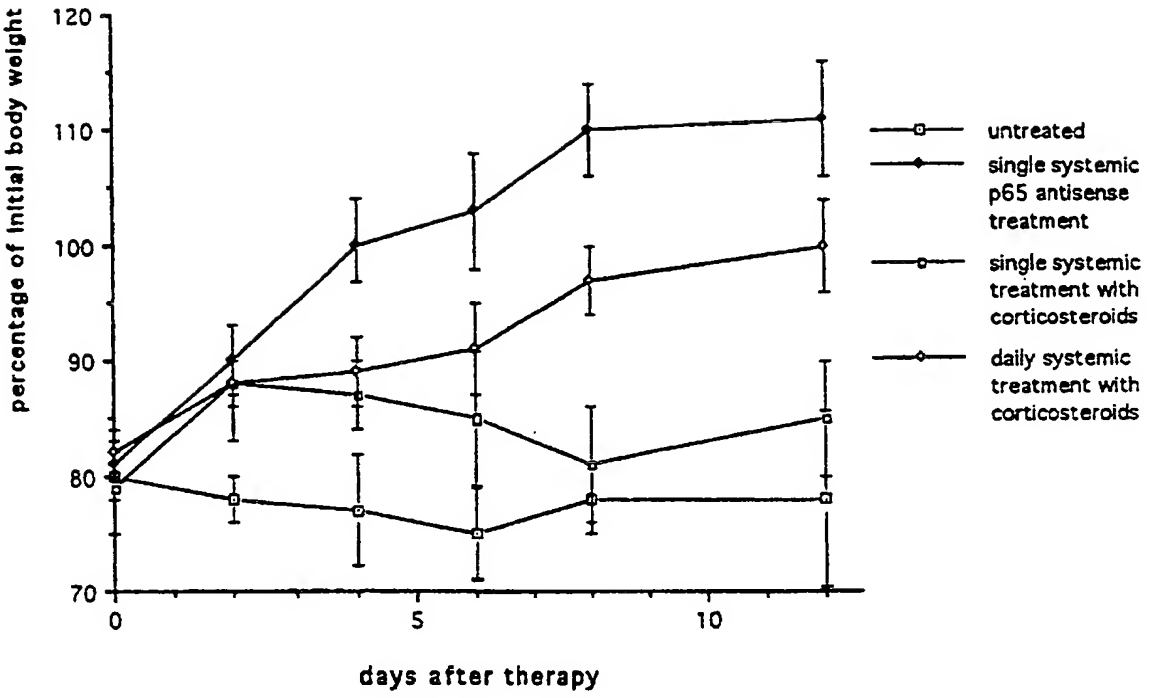

percentage of initial body weight
120
110
100
90
80
70
0
5
10
days after therapy
untreated
single systemic p65 antisense treatment
single systemic treatment with corticosteroids
daily systemic treatment with corticosteroids

Fig. 4b

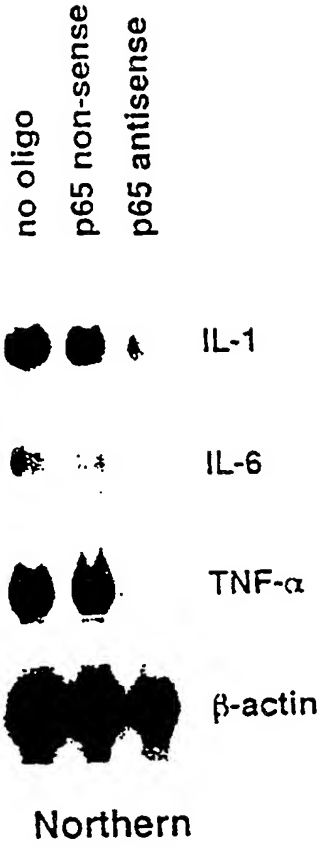

no oligo
p65 non-sense
p65 antisense
IL-1
IL-6
TNF-α
β-actin
Northern

Fig. 5

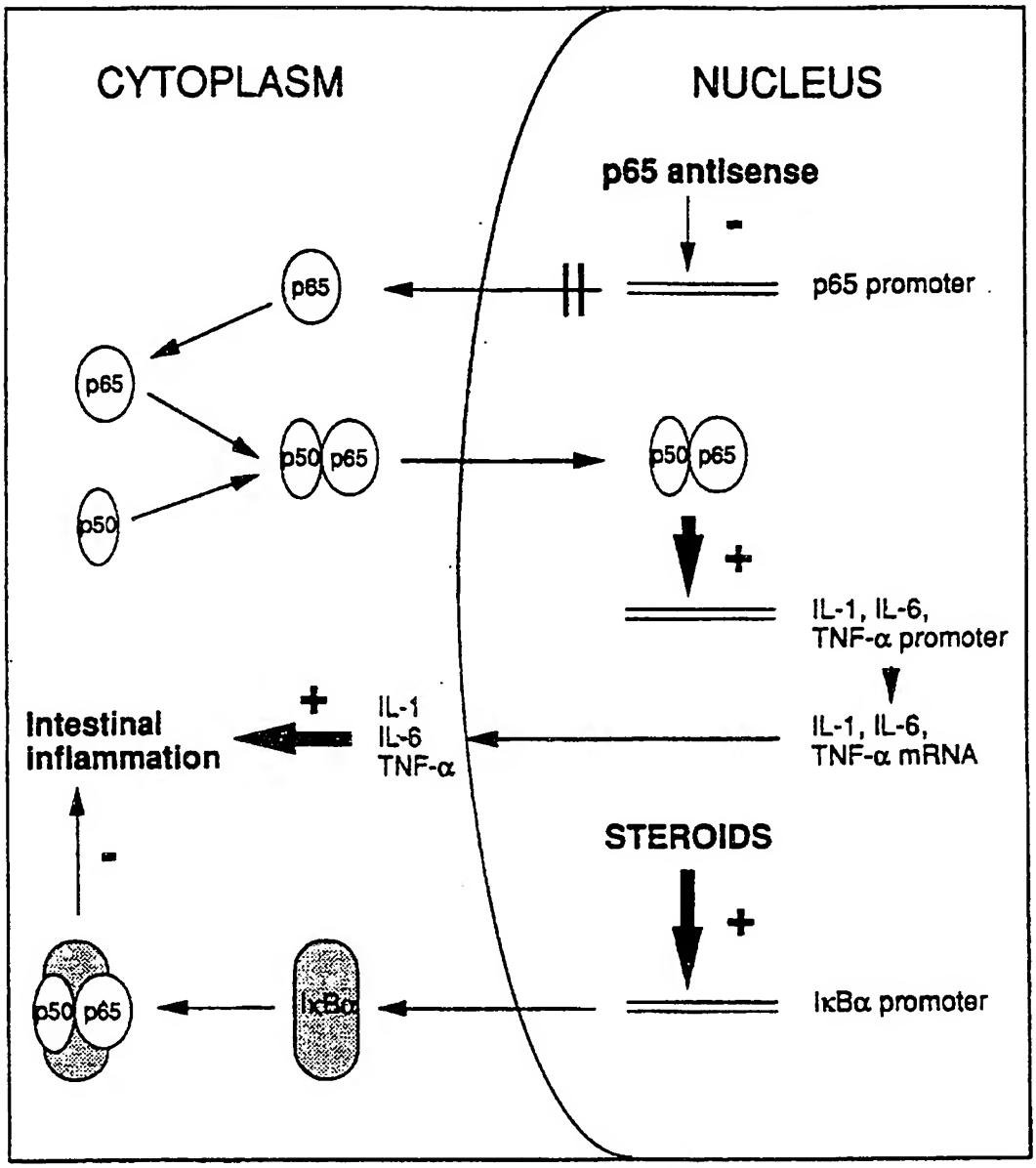

CYTOPLASM
NUCLEUS
p65 antisense
-
p65
p65 promoter
p65
p50 p65
p50 p65
p50
+
IL-1, IL-6,
TNF-α promoter
+
IL-1
IL-6
TNF-α
Intestinal
inflammation
IL-1, IL-6,
TNF-α mRNA
STEROIDS
-
+
p50 p65
IκBα
IκBα promoter

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01019

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 48/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C12N, C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS, MEDLINE, CA, DBA, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Nature Medicine, Volume 2, No 9, Sept 1996, Markus F. Neurath et al, "Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kB abrogates established experimental colitis in mice" page 998 --	1-2,16-18
O,X	European Cytokine Network, Volume 7, No 2, 1996, M.F. Neurath et al, "Local or systemic administration of antisense phosphorothioate oligonucleotides to the P65 subunit of NF-xB abrogates established experimental colitis in mice" page 304 ---	1-2,16-18

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 1997	26 -11- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01019

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9535032 A1 (THE SCRIPPS RESEARCH INSTITUTE), 28 December 1995 (28.12.95), SEQ 6 and the whole document ---	1-2,16-18
X	EP 0589330 A2 (F. HOFFMANN-LA ROCHE AG), 30 March 1994 (30.03.94), SEQ ID No 3 and the whole document --- --------	1-2,16-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01019

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01019

According to PCT rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art.

The claimed invention relates to a pharmaceutical composition comprising a p65 antisense oligonucleotide and a method for diagnosis of inflammatory conditions. A link between the composition and the method could be the nucleotide 5'-GGAACAGTTCGTCCATGGC-3', known from WO95/35032 or the over-expression of p65 in inflammatory conditions, known from Neurath et al.6th Intl. TNF Congress, see search report. Since both the proposed unifying links are known in the art and no other unifying link was found, the application fails to comply with PCT Rule 13.2 *a posteriori*. The following inventions were found:

Invention 1, claims 1-2 and 16-18: Composition comprising the oligonucleotide 5'-GGAACAGTTCGTCCATGGC-3' and its use.
Invention 2, claims 3-15: Method for diagnosis of inflammatory conditions in humans.

The search has been restricted to claim 1.

INTERNATIONAL SEARCH REPORT
Information on patent family members

01/10/97

International application No.
PCT/SE 97/01019

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9535032 A1	28/12/95	AU 7671194 A	15/01/96
EP 0589330 A2	30/03/94	AU 673537 B	14/11/96
		AU 4743093 A	31/03/94
		CA 2105595 A	24/03/94
		CN 1084564 A	30/03/94
		JP 6209778 A	02/08/94
		NZ 248691 A	27/01/95
		US 5591840 A	07/01/97
		ZA 9306838 A	23/03/94